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A Therapeutic Approach for Prostate Cancer

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13. ABSTRACT *(Maximum 200 words)*

Despite significant research in the field of prostate cancer (PCa), the disease remains elusive in terms of its etiology, pathophysiology and clinical management. Identification of new genotypic and phenotypic markers underlying initiation, promotion and endocrine therapy failure would be of paramount significance. We have shown that metallothionein (MT) can induce mitogenic response in tumor cells and that its inhibition can lead to growth arrest and induction of apoptotic cell death. The objective of this training grant was to establish an antisense MT adenovirus construct and test its efficacy on growth inhibition and induction of apoptosis *in vitro* and *in vivo*. Due to experimental difficulties and the short time-frame specified for this award (6 months) we were able only to generate the antisense MT adenoviral construct. The experiments testing the efficacy of this novel construct are ongoing. The significance of this construct lies on the fact that MT is a mitogenic protein and its abundance, as shown in many human primary tumors, is associated with development of drug resistance and poor prognostic outcome. Thus, it is anticipated that this novel antisense construct will have a significant impact on growth inhibition and enhancement of efficacy of chemotherapeutic agents.

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Qasim S. Abdel-Majeed 8/12/99

PI - Signature Date

(3) Table of Contents

(5) Introduction:

Prostate cancer (PCa) still poses question regarding its etiology, pathology, pathogenesis and clinical management. It has been estimated that there may be quarter of a million cases of PC world-wide (1) and the incidence of PC is projected to increase significantly (2). While earlier reports estimated that PC account for 35,000 deaths annually in Europe (3) and 38,000 in the USA (4), recent reports demonstrated that the difference between the incidence rate and the death rate is striking; in 1996 there were 317,000 newly diagnosed PC cases and 41,400 deaths in the USA compared to the 1995 figures of 244,000 and 40,4000, respectively (5). However, it should be noted that there have been no major changes in mortality and no major advances in treatment parallel with the current rise in diagnosis since the introduction of androgen down-regulation regimen. Recent figures on prostate cancer suggest that the incidence vary considerably by race and age (6, 7). It has been shown that the mortality rates for African-American men were 2.2 times higher than those for white men. In more details, addition, the mortality rate among African-Americans has increased 68% between 1960-62 and 1990-92, and in recent years, have increased approximately 4% annually (8).

Despite significant research in the field of PCa, the disease remains elusive in terms of its pathogenesis and clinical management. Identification of new genotypic and phenotypic markers and characterization of molecular mechanisms underlying promotion of PCa to metastatic phenotypes and endocrine therapy failure would be of paramount significance. Several lines of evidence demonstrate a relationship between metallothionein (MT) expression in various types of human primary tumors and disease progression, metastasis, and poor prognostic outcome. MTs are a set of ubiquitous, low molecular weight (6-7kD) aromatic amino acid-free proteins that have 20 reduced cysteine residues which can chelate seven bivalent heavy metal ions through mercaptide bonds (9). In humans, MTs are encoded by a family of genes located at 16q13 (10,11) that contains 10 functional and 7 nonfunctional isoforms (12,13). The gene sequence and expression of all the functional human MT genes have been identified and well characterized: MT-IIA (14), MT-3 (15), MT-4 (16), MT-IA (17), MT-IB (18), MT-IE, MT-IF and MT-IG (19), and MT-IH and MT-IX (20).

MT overexpression has been shown to be associated with disease progression, metastasis, and poor prognostic outcome in a number of human primary tumors, including malignant melanomas (21), ovarian tumors (22), testicular germ cell tumors (23), bladder transitional cell carcinomas (24), and bladder cancer and renal cell carcinoma (25). However, the functional role of MT in tumor cell growth is poorly understood. To answer this question, we have recently demonstrated in breast carcinoma MCF-7 cells that MT overexpression enhances cellular proliferation and *c-myc* gene expression and suppresses *c-fos* and *p53* transcripts, whereas antisense down-regulation of MT induces growth arrest and apoptotic cell death (26). We also showed that the activation of transcription factor NF-kappa B and its interaction with MT may be required for the MT-mediated mitogenic response (27).

In our laboratory, we demonstrated the isoform-specific expression of MT in PCa and further examined the extent to which they modulate disease progression and metastasis. Specifically, our preliminary data using RT-PCR have shown enhanced MT-IIA, MT-IE, and MT-IF gene expression in human PCa biopsy samples when compared to tissues from benign prostatic hyperplasia (BPH), suggesting that at least the aberrant expression of specific MT isoforms(s) may be associated with growth promotion and metastatic disease. This observation was confirmed by IHC localization showing enhanced MT protein expression in cancer cells, basement membrane and lumen secretory products in biopsy specimens from PCa patients when compared to BPH specimens. Our preliminary data also indicated that MT-IIA overexpression alone has enhanced cell multiplication, collagenase (MMP-2) and MSA in a panel of PCa cell lines, LNCaP, PC-3, and DU-

145. These preliminary data have led us to hypothesize that MT plays a pivotal role in the promotion of neoplastic cell growth and in the progression of PCa to metastatic disease. Therefore, in this proposal we seek to ascertain whether the down-regulation of MT gene by antisense adenoviral construct will be able to inhibit growth and metastasis of prostate cancer cells.

(II) Objectives: To further test this hypothesis we proposed the following specific aims: (a) to construct an MT antisense adenoviral vector for achieving high transfection efficiency in human prostate cancer cells, LNCaP, DU-145 and PC-3; (b) to determine *in vitro* growth inhibitory effects of the MT antisense adenoviral construct ; (c) to determine the MT antisense induced apoptosis by quantitative DNA fragmentation and TUNNEL methodology; (d) to monitor tumor growth delay and modulation of metastasis in athymic nude mice after transplantation of transfected cells.

(6) Body:

a. Optimization of trasfection efficiency of prostate cancer cells with adenoviral vectors.

Adv5-β-galactosidase reporter construct was generously provided by Dr. David T. Curiel, University of Alabama at Birmingham, AL. Subconfluent cultures of rrostate cancer cell lines, DU-145, PC-3 and LNCaP, were exposed to various concentrations of the reporter construct (10-100 MOI) and transfection efficiency was determined 24 hr post-transduction by measuring β-galactosidase activity *in situ*. Briefly, the cells were washed in phosphate buffered saline (PBS) and fixed (formaldehyde/formaldehyde solution) at room temperature for 5 min. Cells were then stained (potassium ferricyanide/ferrocyanide, $MgCl_2$ and X-gal) 2 hr to overnight at 37°C , washed in PBS and observed on an inverted microscope. Efficiency of trasfection was measured by scoring dark blue cells positive. The results demonstrated that 100 MOI is optimum for achieving 95-100% transfection efficiency in all cell lines. The results are depicted in Figure 1.

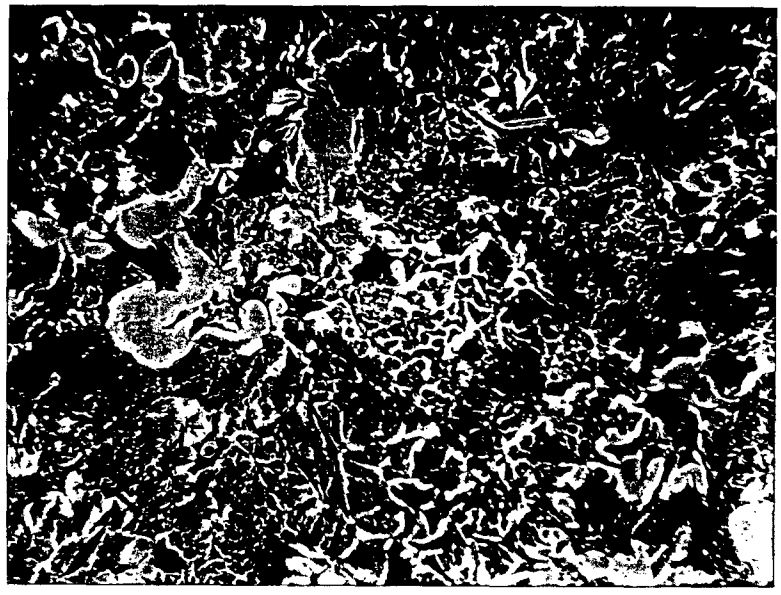
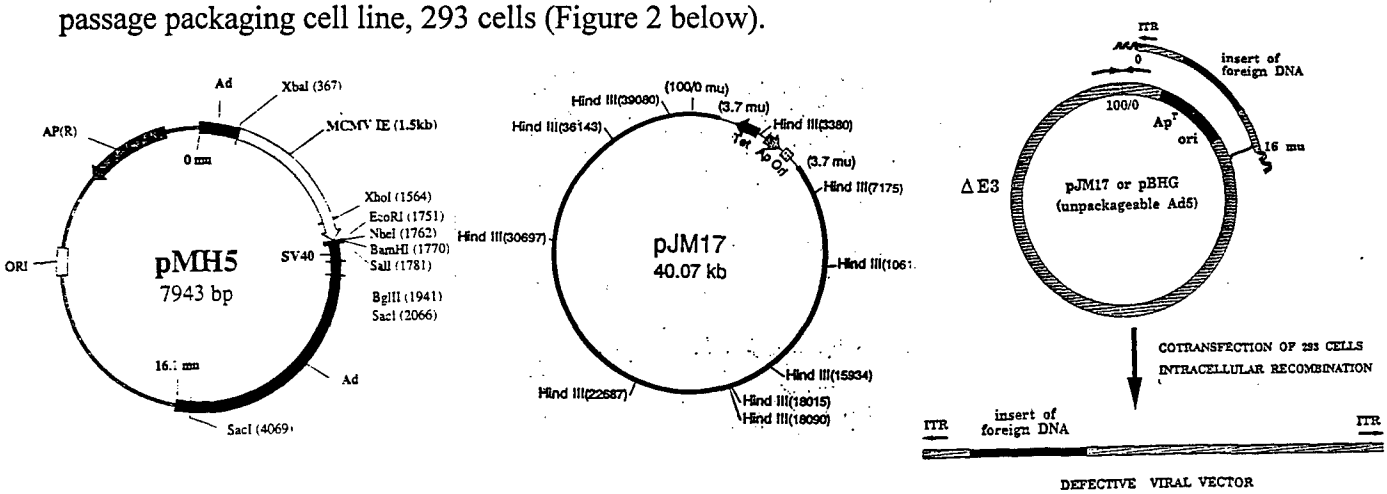


Figure 1: Subconfluent cultures of PC-3 cells were transfected with Adv5-β-gal vector (100 MOI) for 24 hr. Cells were then fixed and stained for β-galactosidase activity *in situ* as shown above. The results demonstrated that adenoviral constructs can efficiently express a transgene (95-100%) in prostate epithelial cells.

b. Construction of an MT Antisense Adenoviral Vector.

Plasmids for adenovirus vector construction for transgene insertion in the E1 region of Ad5 vectors was purchased from Mirobix Biosystems Inc, Ontario, Canada. The kit is composed of the shuttle vector, pMH5, the circular replication-defective Ad genome plasmid pJM17A and the low-passage packaging cell line, 293 cells (Figure 2 below).

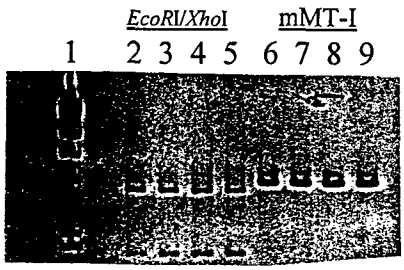


(a) (b) (c)

Figure 2: Adenovirus construction system: shuttle plasmid pMH5 (a) and replication-defective circular viral vector pJM17 (b); intracellular recombination (c)

5'*Eco*RI /3'*Xho*I' fragment encompassing the cDNA of mouse MT-I was initially digested with the restriction enzymes and subsequently removed from agarose gel by electroelution using Bio-Rad electroeluter (Figure 3). To subcloned the digested fragment in reverse (or antisense) or the sense orientation (control) in the E1 region of shuttle plasmid, 5 micrograms of the pMH5 plasmid (purchased from Microbix Biosystems, Inc, Ontario, Canada, see map below) was initially digested with *Eco*R1 and subsequently dephosphorylated using calf intestinal phosphatase (CIP) enzyme to prevent re-ligation. Ligation was carried out in accord with manufacture's instructions (Promega) using 1X buffer containing dATP and 3 U of T4 DNA ligase in 50 µl final volume at 16°C overnight. The reaction was stopped by heating at 75°C for 10 min. The overhangs were then filled using Klenow fragment (1 U) (Promega) in presence of dNTPs at 30°C for 10 minutes and reaction was stopped by heating at 75°C for 10 min. Blunt end ligation was carried out as shown above in presence of 6 U of T4 DNA ligase overnight. Reaction was stopped and samples were stored at -20°C until used.

Figure 3:



Lane 1, λ-Hind-III marker; lanes 1-4, 5'*Eco*RI/*Xho*I3' digest; Lanes 5-8, mMT-I

b. Transformation.

Competent *E coli* $DH_{5\alpha}$ was transformed with plasmids in accord with manufacturer's instructions. Briefly, bacterial cells were preincubated in ice with recombinant or control plasmids for 30 minutes prior to temperature shock at 42°C for 1.5 min. Bacterial cells were then grown in 1 ml LB with (shaking at 200 cpm) in antibiotic-free medium for 1 hr at 37°C. Cultures were then transferred into flasks containing100 ml LB supplemented with ampicillin (50 μg/ml) and subsequently grown for an additional 24 hr. Bacterial cell growth was detected spectrophotometrically. Samples of culture media were further cultured in LB agar plates containing ampicillin at 50 μg/ml. Individual colonies (20) picked and further grown in LB agar plates.

c. Isolation of plasmid DNA .

Mini preps were used for individual clones by culturing bacteria in 10 ml LB media containing ampicillin overnight. DNA was isolated using standard methods. Briefly, Harvested overnight cultures were pelleted, washed in TE buffer, resuspended in GTE buffer containing lysozyme and incubated at 30°C for 20 min. Cell were lysed in presence of SDS and NaOH and plasmid DNA was isolated by centrifugation in presence of 3M K^+ 5M OAc, pH 5.6. Ribonucleic acids were then removed using RNase A at 37°C for 30 min. DNA was extracted twice using phenol/chloroform/isoamyl alcohol (24:24:1) and precipitated with 1 volume of isopropanol. The pellets were washed once in 70% ethanol and stored at -20°C for further analysis.

d. Analysis of recombinant plasmids by restriction mapping.

To determine whether our DNA inserts (sense and antisense) were successfully cloned in the shuttle plasmid pMH5, we analyzed the DNA plasmids using 0.7% agarose gel containing ethedium bromide.

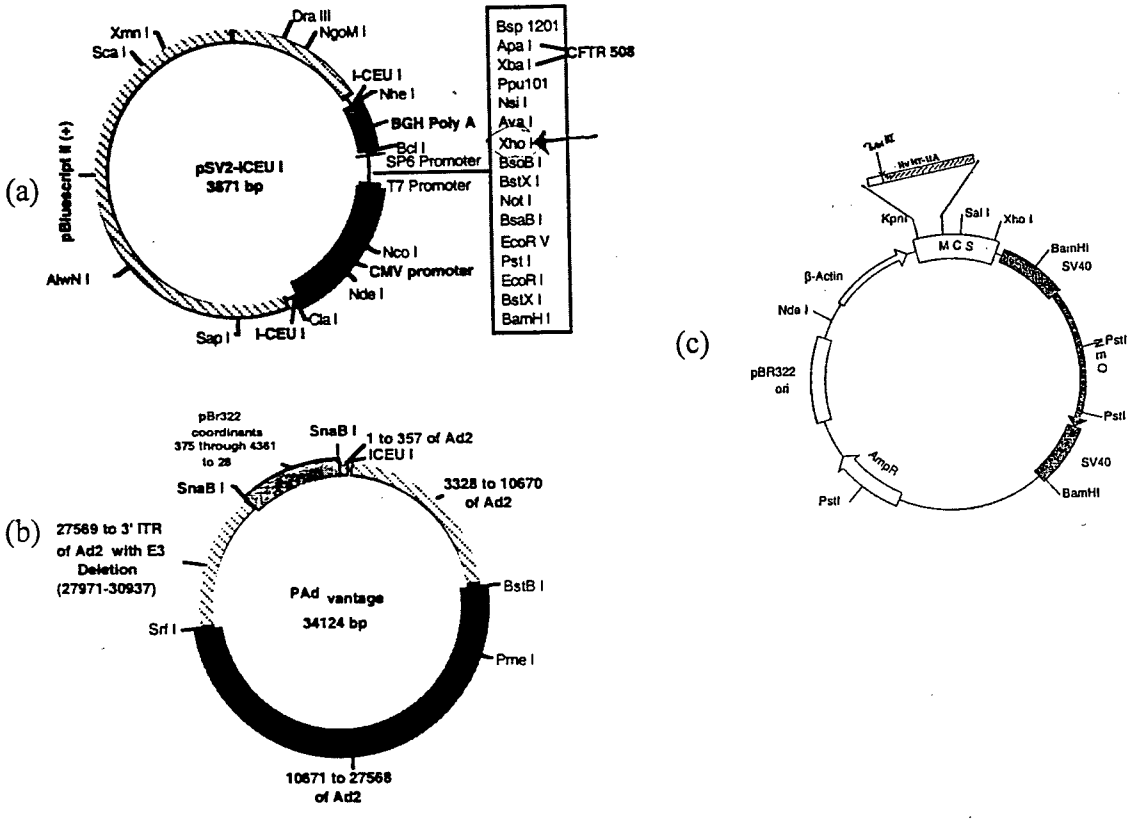
e. Generation of recombinant shuttle plasmids. Our results showed that among the twenty clones picked up for analysis of DNA inserts, none had shown a band or a band shift in the gel, as opposed to controls. We then decided to repeat the subcloning, transformation and analysis steps shown above without any successful attempt to subclone the mMT-I fragment. Our results have indicated that the bacteria were successfully cloned with the control plasmids, but not with our recombinant shuttle plasmids.

Even though we scrutinized all of our steps to subclone the DNA inserts we were unable to detect why this system have failed (see results below). We concluded that, the shuttle plasmid we purchased may have defective multiple cloning site at the EcoR1 site.

f. Use of $pAd_{vantage}$ System for construction of MT antisense vector.

Our next approach was to use an alternative system for cloning hMT-II inserts in both orientation. A novel cloning method for recombinant adenovirus construction in Escherichia coli has been developed by Genzyme Co, Framingham, MA) (28). The $pAd_{vantage}$ system was a generous gift by Dr. D. W. Souza Genzyme Corporation. The system is composed of the shuttle plasmid pSV-ICEU I (containing the cytomegalovirus promoter (CMV), multiple cloning site (MCS) and polyadenylation site)(Figure 3a) and the viral vector $pAd_{vantage}$ (Figure 3b). Because of close homology between human metallothionein and the use of human prostate cancer cells, we decided to use human MT-IIa cDNA (pBAcNEO-sMT-IIa) to generate the antisense adenovirus constructs (Figure 3c).

Figure 3: $pAd_{vantage}$ system: (a) the shuttle plasmid pSV-ICEU I; (b) $pAd_{vantage}$ viral vector; and (c) pBAcNEOsMT-IIa.



g. Isolation of hMT-IIa from pBAcNEO-sMT-IIa plasmid.

Twenty microgram of pBAcNEO-sMT-IIa plasmid was initially digested with 3'*Xho*I- *Kpn*I5' for 2 hr at 37°C. The products were then fractionated into a 0.7% agarose gel containing ethedium bromide. A DNA fragment (approx 300 bp) encompassing hMT-IIA was then isolated using electroelution technique in accord with the manufacturer instructions (Figure 4). Briefly, gel slices containing the DNA were placed in an apparatus equipped with tubes capped with special membranes and DNA was collected in the membranes by electroelution and subsequently precipitated using standard techniques. The cDNA was then stored at -20°C until used.

and extension at 59°C for 15 sec. The products were then fractionated into a 0.7% agarose gel containing ethedium bromide. A DNA fragment (approx 300 bp) encompassing hMT-IIA was then isolated using electroelution technique in accord with the manufacturer instructions (Figure 4). Briefly, gel slices containing the DNA were placed in an apparatus equipped with tubes capped with special membranes and DNA was collected in the membranes by electroelution and subsequently precipitated using standard techniques. The cDNA was then stored at -20°C until used.

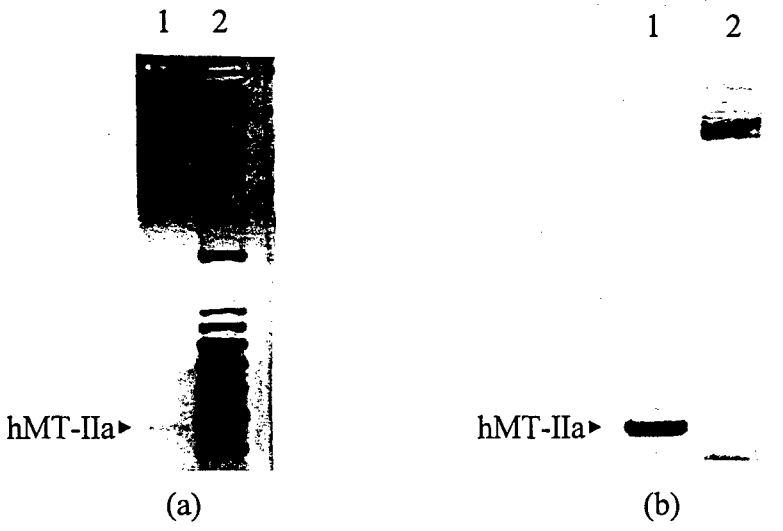


Figure 4: (a) Agarose gel electrophoresis (0.7%) showing 3′*Xho*I- *Kpn*I5′ hMT-IIa fragment (approximately 320 bp); lane 1 3′*Xho*I- *Kpn*I5 double digest, lane 2 DNA marker ; (b) PCR amplification of hMT-IIa using pBAcNEOs-MT-IIa as a template, lane 1, hMT-IIa; lane 2, pBAcNEOs-MT-IIa plasmid

h. Cloning of hMT-IIa in the shuttle plasmid pSV-ICEU I.

Preparation of the shuttle plasmid:

The shuttle plasmid pSV-ICEU I was initially digested with *Xho*-I, dephosphorylated with calf intestine phosphatase (CIP) to prevent re-ligation, and precipitated with phenol/chloroform in accord with standard techniques and the stored -20°C until used.

Ligation:

For preparation of the antisense construct, the 3′*Xho*I- *Kpn*I5′ fragment encompassing the human MT-IIa was initially ligated to the shuttle plasmid pSV-ICEU I using 1X ligation buffer, 3 U of T4 DNA ligase, 0.25 μg of shuttle plasmid with three-fold molar excess of the DNA insert at 15°C overnight. The reaction was stopped at 75°C for 10 min. The overhangs were then filled with Klenow fragment (1 U) (Promega) in presence of dNTPs at 30°C for 10 minutes and reaction was stopped by heating at 75°C for 10 min. Blunt end ligation was carried out as shown above in presence of 400 U of T4 DNA ligase (New England Biotechnology) overnight. The reaction was stopped and samples were stored at -20°C. For the preparation of the control sense plasmid, both *Xho*I-digested pSV-ICEU I and 3′*Xho*I- *Kpn*I5 fragment were initially filled using Klenow fragment as shown above to generate blunt ends and ligation was carried out using high concentration of T4 DNA ligase (New England BioLabs) at 15°C overnight.

Transformation and plasmid Isolation:

Competent *E coli* $DH_{5\alpha}$ was transformed with plasmids in accord with recommended procedure as described above. Mini preps were used for isolation of individual clones in accord with standard protocol as shown above.

Analysis of ligation:

The fidelity of DNA insertion was analyzed using 0.7% agarose gel containing ethedium bromide. The results demonstrated that hMT-IIa was efficiently subcloned in the shuttle vector (Figure 5). Initially, all colonies screened for the control vector have shown no DNA insert and we had repeated the whole cloning techniques before we were successful on isolating a positive clone.

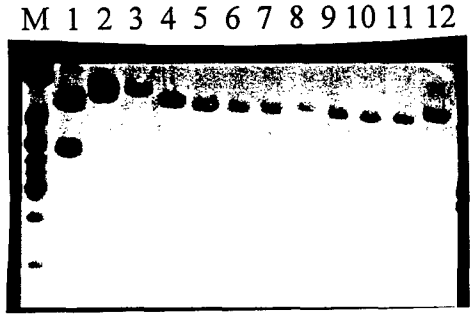


Figure 5: Gel electrophoresis demonstrating a size shift in the recombinant shuttle vector as opposed to control plasmid. M, DNA marker; lane 1-5 MT -IIa antisense recombinant clones; lanes 7-9 control MT-IIa (sense) recombinant clones; lanes 10-12, shuttle plasmid

Analysis of orientation:

The orientation of the DNA insert was performed using restriction mapping of the recombinant plasmids followed by analysis using 0.7% agarose gel electrophoresis. The results demonstrated that the transgene is in the antisense orientation.

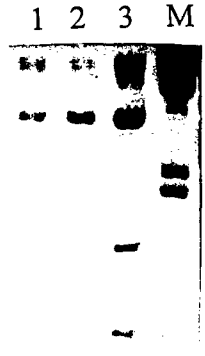


Figure 6: Digestion of the recombinant plasmids with *Sca*I and *Bam*HI. B. It is anticipated that three (lanes 1-3) fragments will be generated following the double digestion in the positive clones. Lane 3 represents a positive clone for the MT antisense clone. M, DNA marker.

Digestion of recombinant plasmid with I-Ceu I:

A fragment encompassing CMV promoter-antisense MT-IIa cDNA and poly A regions was digested from the recombinant shuttle plasmid pSV-ICEU I using I-*Ceu* I (New England Biolabs). The DNA insert was then isolated using agarose gel electrophoresis and electroelution as described above. After precipitation, the pellet was stored at -20°C.

J. Subcloning of the antisense MT-IIa from pSV-ICEU I into the I-*Ceu* I site of the $pAd_{vantage}$ viral vector.

***Preparation of $pAd_{vantage}$ for cloning*:**

Five micrograms of $pAd_{vantage}$ were digested with 30 U of I-*Ceu* I in a total volume of 500 µl of 1 X I-*Ceu* I buffer at 37°C for 3 hr. 10 units were added every hr to reach 30 U by the end of the 3 hr period. The plasmid was then dephosphorylated using CIP as shown above for an additional 20 min at room temperature and DNA was extracted using phenol/chloroform and precipitated with ethanol as shown above.

Subcloning of the I-*Ceu* I fragment into the I-*Ceu* I-site of the $pAd_{vantage}$:

The viral vector (0.25 µg) and three-fold molar excess of the I-Ceu I DNA insert were incubated at 16°C overnight in presence of 400 U of T4 DNA ligase and 1 X ligase buffer in a total volume of 50 µl. The fidelity of the DNA insert in the viral vector was verified by transformation, screening of putative colonies, and by agarose gel electrophoresis when compared to the viral vector alone as we showed above.

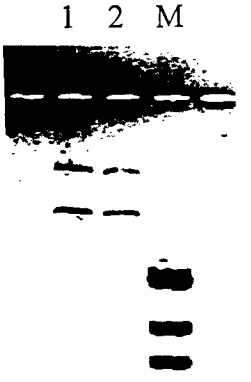


Figure 7: Agarose gel electrophoresis (0.7%, TAE Buffer) for analysis of MT antisense adenovirus construct. A representative clone (lanes 1) was analyzed for efficient ligation of I-*Ceu* I fragment into the I-*Ceu* I-site of the $pAd_{vantage}$ and compared to the control plasmid (lane 2). A shift in band size in lane 1 as opposed to lane 2 indicates successful ligation.

k. 293 Cell Culture.

Low passage 293 cells, a human embryo kidney cells transformed by sheared Adenovirus type 5 DNA, were obtained from Microbix Biosystems Inc., (Ontario, Canada). They contain and express the early region (E1) of Ad 5 virus and can complement the E1 defective Adv mutants and vectors. The cells were grown in MEM supplemented with Earle's salts, glutamine, 10% heat-inactivated FBS, penicillin and streptomycin and incubated at 37°C with 5% CO_2.

l. Viral Packaging in 293 cells.

Preparation of the viral constructs (by first digesting the construct with *Sna*BI to expose the inverted terminal repeats) for packaging in 293 cells is now underway and it is anticipated the viral plaques will become visible in about 7-10 days.

(7) Key Research Accomplishments:

- Generation of novel recombinant antisense MT adenovirus vector.

(8) Reportable outcomes:

- Construction of metallothionein MT adenovirus construct.
- Applied for funding for a DOD grant in 1999 entitled "Antisense down-regulation of metallothionein: a combined therapeutic approach for prostate cancer."
- The data will be also used for funding application to the NIH, ACS and CaPCure.

(9) Conclusions.

The aim of this training grant was to construct a metallothionein antisense adenovirus vector and test its efficacy on enhancing growth arrest and inducing apoptosis in cancer cells using *in vitro* and *in vivo* approaches. - Like other drug resistance genes, metallothionein has been shown to play a role in protecting cancer cells against chemotherapeutic agents and radiotherapy– thus this vector can also be used to enhance sensitization of cancer cells to chemo/radiotherapy. Because this application was a *training* grant (no funds for research supplies or other personnel), and due to the unexpected research problems, I wasn't able to accomplish all specific aims within the short-time frame specified for the grant (6 months). One recommendation, at least, is to include funding for research supplies and to extend the time-frame for these training grants to 1-2 years, so that the research will be completed.

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11. Appendices: N/A

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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